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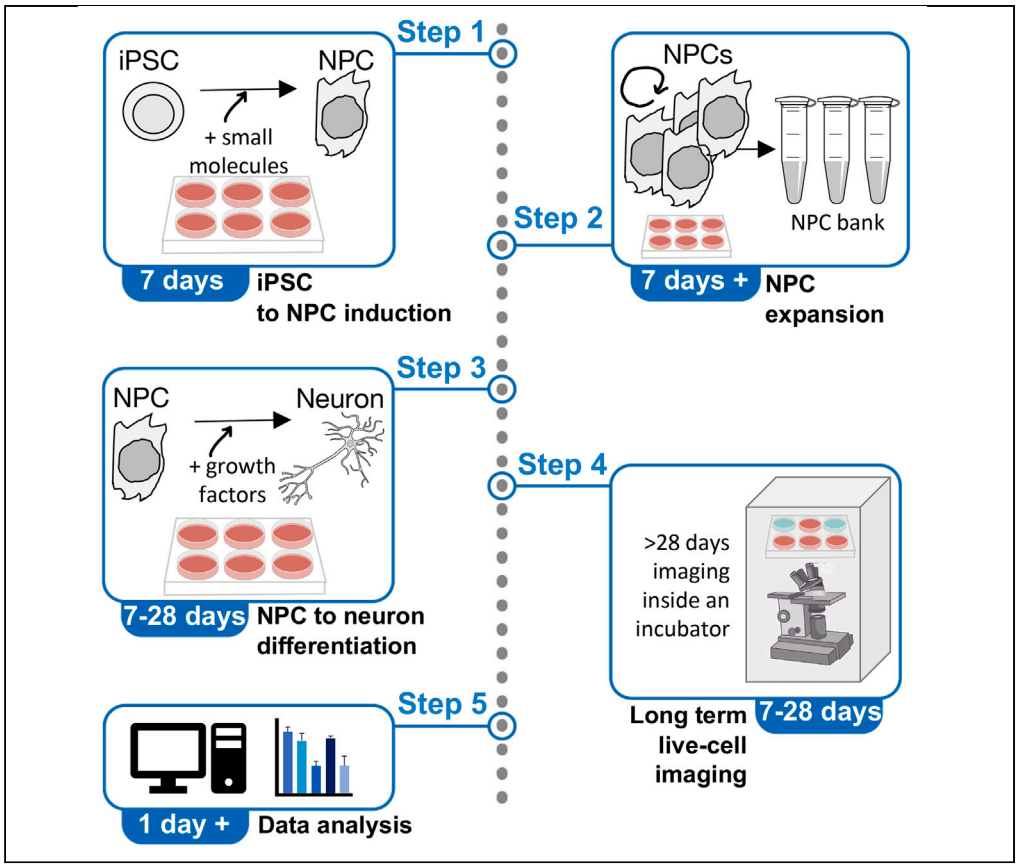
Long-term live cell imaging during differentiation of human iPSC-derived neurons



Live-cell imaging is crucial to appreciate the dynamics and the complexity of cellular interaction processes. However, live-cell imaging of human neurons is challenging due to neuronal sensitivity. Here, we describe a long-term live-cell imaging protocol for neurons derived from human induced pluripotent stem cells. By using an IncuCyte live-cell imaging system, we have obtained information on neuronal dynamics during the different stages of neurogenesis. The protocol has also been developed to monitor the dynamics of the neuronal intracellular organelles.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Jingqi Wang, Paul A. Gleeson, Lou Fourriere

pgleeson@unimelb.edu.au (P.A.G.)
lou.fourriere@unimeb.edu.au (L.F.)

Highlights

Generation of enriched human neurons from iPSCs

Long-term live imaging of neuron differentiation using the IncuCyte system

Live imaging of organelle dynamics over extended periods during neuronal development

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Long-term live cell imaging during differentiation of human iPSC-derived neurons

Jingqi Wang,[1] Paul A. Gleeson,[1,3,*] and Lou Fourriere[1,2,*]

[1]The Department of Biochemistry and Pharmacology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, VIC 3010, Australia

[2]Technical contact

[3]Lead contact

*Correspondence: pgleeson@unimelb.edu.au (P.A.G.), lou.fourriere@unimeb.edu.au (L.F.)
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SUMMARY

Live-cell imaging is crucial to appreciate the dynamics and the complexity of cellular interaction processes. However, live-cell imaging of human neurons is challenging due to neuronal sensitivity. Here, we describe a long-term live-cell imaging protocol for neurons derived from human induced pluripotent stem cells. By using an IncuCyte live-cell imaging system, we have obtained information on neuronal dynamics during the different stages of neurogenesis. The protocol has also been developed to monitor the dynamics of the neuronal intracellular organelles.

For complete details on the use and execution of this protocol, please refer to Wang et al.[1]

BEFORE YOU BEGIN

Institutional permissions

All experiments described below were authorized and follow our Institutional and national regulations. Experiments on pluripotent stem cells must be performed in accordance with the relevant institutional and national guidelines and regulations, which might differ between institutes and countries. Note that some authorization might be required and approved before the work starts.

PC2 procedures need to be followed throughout the protocol. The procedure needs to be performed under a Class II Biohazard hood and with standard personal protective equipment worn. All buffers and reagents need to be maintained in a sterile condition.

This protocol uses well-characterized iPSCs obtained from healthy donors.[2] The protocol can be further extended to patient-derived iPSCs and be adapted to characterize various somatic and dendritic organelles in human neurons. An overview of the timeline and major steps involved in this protocol is shown in the Graphic Abstract.

Preparation of the aliquots

Timing: around 4 h

The culture of induced pluripotent stem cells (iPSCs), neural precursor cells (NPCs), and neurons requires many reagents (coating molecules, medium, small molecules, growth factors) (Figure 1) and it is important to preserve these reagents by minimizing the number of freezing and thawing cycles. Before starting, make sure that all the reagents listed in the key resources table are resuspended,

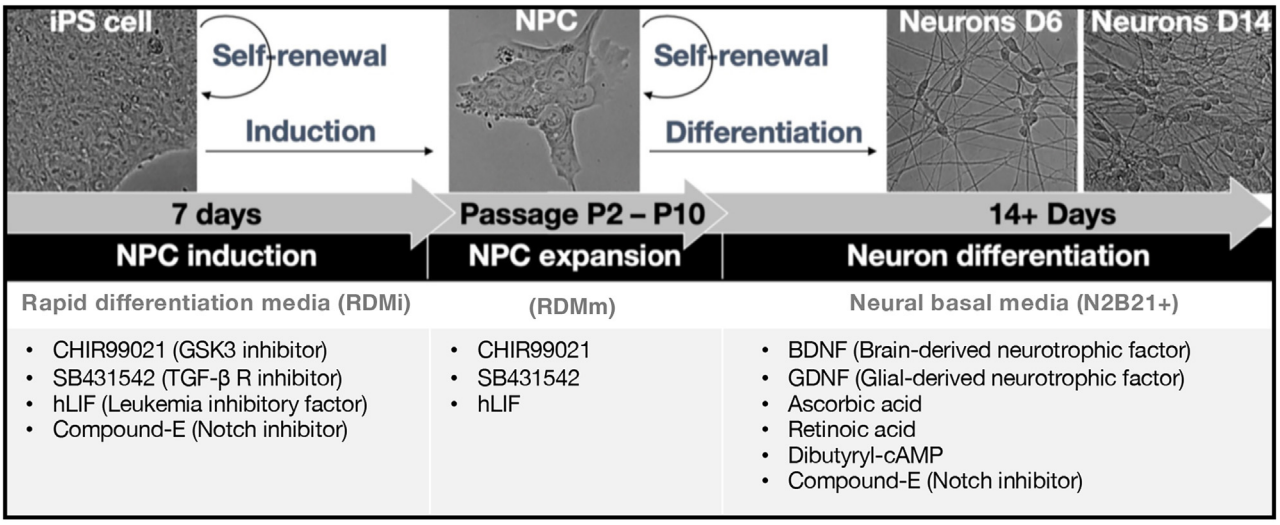

Figure 1. Timeline for the generation of an enriched hiPSC-derived neuron culture

Schematic protocol to generate iPSC-derived human neurons using different small molecules and growth factors supplied at different differentiation stages. IPSCs are first induced into neural precursor cells (NPCs) for 7 days. NPCs can be expanded for a cell bank and differentiated into neurons using neuronal growth factors. Phase contrast images were taken using the FLoid imaging station at the indicated days.

aliquoted and correctly stored. Aliquots can be stored for up to three months following instructions from the manufacturer.

1. Aliquot the coating material:
   a. Matrigel, dilute one aliquot (following instructions from the manufacturer for each stock) in DMEM/F-12, stock 6 mL aliquots at −20°C for coating one 6 well plate (1 mL per well).
   b. Laminin, aliquot into 100 µg aliquots and store at −20°C, each aliquot diluted in 5–10 mL of cold PBS to give 10–20 µg/mL final concentration (7.5 mL of cold PBS was used in this protocol, with 15 µg/mL final concentration).

   ⚠ CRITICAL: Frozen laminin needs to be thawed on ice. Pipette tips need to be pre-cooled in the fridge when handling laminin to prevent gel formation.

   c. Poly-L-orthinine, 50 mL 0.01% w/v (0.1 mg/mL), stored at 2–8°C, 1:5 dilution in ultrapure water to 20 µg/mL.
   d. Vitronectin, 80 or 160 µL aliquots diluted in 2 or 4 mL of CellAdhere dilution buffer (for 2 or 4 wells respectively of a 6 well plate, 1 mL per well).
2. Preparation of aliquots of the supplements.
   a. N2, 500 µL aliquot, −20°C.
   b. B21, 1 mL aliquot, −20°C.
   c. B21 without vitamin A, 1 mL aliquot, −20°C.
   d. Penicillin-Streptomycin, 500 µL aliquot, −20°C.
   e. StemFlex supplement, 5 mL aliquot, −20°C.

   Note: store the labelled component aliquots in the same box inside the freezer as it will be easier to grab all of them while preparing new media.

3. Aliquot the solutions to detach the cells:
   a. Accutase, aliquots of 5 mL, −20°C.
   b. ReLeSR, aliquots of 10 mL, 20–25°C.
4. Aliquot the small molecules:

Note: Manufacture instructions need to be followed for aliquot preparation and optimal storage. Resuspended aliquots need to be stored between −20°C to −80°C. We routinely stored our aliquots at −20°C for up to three months.

a. CHIR99021, 1 mg is resuspended in 215 μL DMSO to give a stock concentration of 10 mM; aliquots of 10 μL stored at −20°C.
b. SB431542, 1 mg is resuspended in 260 μL of DMSO to give a stock concentration of 10 mM, aliquots of 10 μL stored at −20°C.
c. LIF, 10 μg is resuspended in 100 μL of sterile water to give a stock concentration of 100 ng/μL, aliquots of 10 μL stored at −20°C.
d. Compound E, 500 μg is resuspended in 1020 μl of DMSO to give a stock concentration of 1 mM, aliquots of 10 μL, stored at −20°C.
e. BDNF, 10 μg is resuspended in 100 μL of ultra-pure water to give a stock concentration of 100 ng/μL, aliquots of 10 μL, stored at −20°C.
f. GDNF, 10 μg is resuspended in 100 μL of water to give a stock concentration of 100 ng/μL, aliquots of 10 μL stored at −20°C.
g. db-cAMP, 25 mg is resuspended in 254 μL of water to give a stock concentration of 200 mM, aliquots of 25 μL stored at −20°C.

Note: db-cAMP needs to be protected from the light.

h. AA, 500 mg is resuspended in 2800 μL of water to give a stock concentration of 1M, aliquots of 200 μL stored at −20°C.
i. DAPT, 5 mg is resuspended in 575 μL of DMSO to give a stock concentration of 20 mM, aliquots of 50 μL stored at −20°C.
j. ROCK inhibitor Y27632, 1 mg is resuspended in 312 μL of ultrapure water to give a stock concentration of 10 mM, aliquots of 20 μL stored at −20°C.

Note: Stock of laminin, BDNF, GDNF and LIF need to be keep at −80°C before aliquoting.

Note: Spin down stock tube before aliquoting any solution.

Note: Use PCR tubes to aliquot small aliquot volumes of the small molecules and growth factors.

Note: Aliquots can be stored in a box to easily find them when needed.

⚠ CRITICAL: Manufacture instructions need to be followed for the reconstitution and storage of each component. It is important to avoid repetitive freezing/thawing cycles.

Media preparation

Timing: 1 h

5. Preparation of the StemFlex iPSC media.
   a. Mix 5 mL of the Stemflex complement into 45 mL of StemFlex media as described in the corresponding table under the materials and equipment section.
6. Preparation of the RDM media.
   a. Mix the components as described in the corresponding table below under the materials and equipment section.

Note: only prepare small volume of fresh RDM (ie: 50 mL) instead of preparing 500 mL.

Note: Pool one aliquot tube of each small molecule in a 10-mL Falcon tube; one Falcon tube for the NPC induction/maintenance (with CHIR, SB, LIF, C-E, Y27632) and one Falcon tube for the neuron culture (BDNF, GDNF, AA, db-cAMP, C-E, Y27632). It is then convenient to access the mix of reagents during the preparation of the media every day.

7. If starting directly from NPCs, prepare the N2B21 media.
8. Mix the components as described in the corresponding table under the materials and equipment section.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Matrigel	Corning	Cat #354277
Laminin	Gibco	Cat #230170015
Poly-L-ornithine	Sigma	Cat #P4957
Vitronectin	STEMCELL technologies	Cat #07180
CellAdhere Dilution Buffer	STEMCELL technologies	Cat # 07183
StemFlex Medium	Thermo Scientific	Cat # A3349401
DMEM/F12 medium	Gibco	Cat #11320-082
Neural basal medium (NBM)	Gibco	Cat #21103-049
N2 supplement (N2)	Lifetech	Cat #17502048
B21 supplement (B21)	Miltenyi Biotec	Cat #130-093-566
B21 supplement without vitamin A	Miltenyi Biotec	Cat #130-097-263
GlutaMAX	Lifetech	Cat #35050061
Penicillin-Streptomycin (Pen/strep)	Sigma	Cat #P4333-100ML
Bovine serum albumin (BSA)	Sigma	Cat #A9576
Accutase	STEMCELL technologies	Cat #07920
ReLeSR	STEMCELL technologies	Cat #100-0484
Y27623 (ROCK inhibitor)	STEMCELL technologies	Cat #72302
CHIR99021 (CHIR)	STEMCELL technologies	Cat #72052
SB431542 (SB)	STEMCELL technologies	Cat #72232
Leukemia inhibition factor (LIF)	STEMCELL technologies	Cat #78055.1
Compound E (C-E)	STEMCELL technologies	Cat #73952
Brain-derived neurotrophic factor (BDNF)	STEMCELL technologies	Cat #78805
Glial-derived neurotrophic factor (GDNF)	STEMCELL technologies	Cat #78058
Ascorbic Acid (AA)	STEMCELL technologies	Cat #72132
Dibutyryl-cAMP (db-cAMP)	STEMCELL technologies	Cat #73882
CryoStor CS10	STEMCELL technologies	Cat #07930
5-Fluoro-2′-deoxyuridine (5-FdU)	Sigma	Cat #F0503-100MG
BODIPY TR Ceramide Golgi Staining Kit	Abcam	Cat #ab269449
Experimental models: Cell lines		
Human iPSC: WAB00005, healthy, F, 62 yrs	Alice Pébay	Wang et al.[1]
Other		
IncuCyte Live-Cell Analysis System	Sartorius	N/A
Incubator for the IncuCyte Live-Cell Analysis System	N/A	N/A
Dedicated incubator for the stem cells	N/A	N/A
µ-Slide 8-well high glass bottom	iBidi	Cat #80807
Costar 6 Well Clear Not Treated Multiple Well Plates with Lid, Sterile	Sigma-Aldrich	Cat #CLS3736-100EA

⚠ CRITICAL: iPSCs, NPCs and neurons are sensitive to temperature and CO_2 variation. Cell viability will be drastically affected if an incubator is heavily used by laboratory staff and/or if the incubators are not sufficiently accurate to deliver constant temperature and CO_2).

MATERIALS AND EQUIPMENT

Coating

Reagent	Final concentration	Amount
Vitronectin	-	40 μL per well (6 well plate)
Cell Adhere dilution buffer	-	1 mL per well (6 well plate)
Matrigel	-	1 mL per well (6 well plate)
Laminin	10–20 μg/mL	250 μL per well (24 well plate)
Poly-L-ornithine	20 μg/mL	250 μL per well (24 well plate)

Detaching

Reagent	Final concentration	Amount
ReLeSR	As stock	1 mL per well (6 well plate)
Accutase	As stock	500 μL per well (6 well plate)

StemFlex iPSC medium

Reagent	Final concentration	Amount
StemFlex basal medium (DMEM/F-12)	-	45 mL
StemFlex supplement	-	5 mL
Total	-	**50 mL**

Rapid Differentiation medium (RDM) basal medium

Reagent	Final concentration	Amount
DMEM/F-12	-	23 mL
NBM	-	23 mL
N2	1X	500 μL
B21 without vitamin A	1X	1 mL
GlutaMAX	1X	500 μL
Pen/Strep	1X	500 μL
BSA	1%	2 μL
Total	-	**50 mL**

Small molecules for NPC induction (RDMi)

Reagent	Final concentration	Amount
RDM basal medium	-	10 mL
CHIR	4 μM	4 μL
SB	3 μM	3 μL
LIF	10 ng/mL	1 μL
C-E	100 nM	1 μL
Total	-	**10 mL**

Small molecules for NPC maintenance (RDMm)

Reagent	Final concentration	Amount
RDM basal medium	-	10 mL
CHIR	3 μM	3 μL
SB	2 μM	2 μL
LIF	10 ng/mL	1 μL
Total	-	**10 mL**

Neuronal basal medium (N2B21)		
Reagent	Final concentration	Amount
NBM	-	48 mL
N2	1X	500 µL
B21	1X	1 mL
GlutaMAX	1X	500 µL
Pen/Strep	1X	500 µL
BSA	1%	2 µL
Total	-	**50 mL**

Growth factors for neuronal medium (N2B21+)		
Reagent	Final concentration	Amount
N2B21 basal medium	-	10 mL
BDNF	20 ng/mL	2 µL
GDNF	20 ng/mL	2 µL
Ascorbic Acid	200 µM	10 µL
db-cAMP	200 µM	2 µL
C-E	200 nM	2 µL
Total	-	**10 mL**

Note: Basal media (StemFlex, RDM and N2B21) should be kept at 4°C for no longer than 2 weeks. Media with small molecules or growth factors added (RDMi, RDMm and N2B21+) should be freshly made up and used up for each experiment on the day.

⚠ CRITICAL: It is important to always preserve the quality of the media, therefore it is recommended to aliquot the exact volume needed and to keep the media bottle stock in storage at 4°C (ie: don't keep the bottle on the bench or under the hood for an extended period).

⚠ CRITICAL: Small molecules and growth factors should be kept frozen in small aliquots (to preserve them from repeated thawing and freezing cycles).

⚠ CRITICAL: Add fresh aliquots to the basal medium on the day of experiment or media changing.

Alternative: B27 supplement can be used as an alternative to the B21 supplement (with and/or without vitamin A). B27 and B21 contain similar ingredients, except that B21 does not contain biotin. B21 is recommended in biotin-sensitive studies. N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) can be used as an alternative Notch/γ-secretase inhibitor to Compound E (C-E) throughout.

STEP-BY-STEP METHOD DETAILS

iPSC to NPC induction

Timing: 7 days

The first step is to have optimal iPSCs in cell culture before the induction of the neural precursor cells (NPCs) from iPSCs begins. We have adopted an established protocol[3] to induce iPSCs into NPCs. The iPSCs to NPCs induction takes 7 days.

⚠ CRITICAL: iPSCs are from a frozen stock and need to be passaged at least once before the induction is initiated (around 2 weeks for iPSC thawing and maintenance).

1. Thaw iPSCs from a frozen stock.
   a. Thaw the vitronectin aliquot at 20–25°C and prepare for each well of a 6 well vessel (10 cm^2) 40 μL of vitronectin diluted in 1 mL of Cell Adhere dilution buffer.
   b. Coat culture vessels with vitronectin and incubate at 20–25°C for 1 h.

   Note: The temperature range 20–25°C corresponds to the room temperature (or RT) conditions.

   ⚠ CRITICAL: It is essential to use non-tissue culture treated culture plate for vitronectin coating.

   Alternative: Matrigel can be used as an alternative to vitronectin. See point 3.a for Matrigel coating instructions.

   c. Prepare complete StemFlex media with the Rock Inhibitor Y27632 (5 μL of 10 mM Y27632 stock in 10 mL for a final concentration of 5 μM).

   ⚠ CRITICAL: The ROCK inhibitor (Y27632) is only added for the first 24 hours of culture (5 μL in 10 mL) to promote cell survival.

   d. Incubate the frozen stock of iPSCs in a 37°C water bath until the ice in the tube disappears.
   e. Transfer the thawed iPSCs in an empty 10-mL Falcon tube and then add 4 mL of StemFlex media to the iPSCs drop-wise while shaking the tube to avoid an osmolarity shock and to obtain the iPSC suspension.
   f. When ready, aspirate the vitronectin from each well of the 6 well plate and replace with fresh StemFlex media, 2 mL per well.
   g. Add 100 μL of iPSCs suspension to one coated well as a starting point.
   h. Check the cell density and add more iPSC suspension if needed to achieve an optimal cell density (see Figures 2A and 2B).
   i. Maintain the iPSC culture plate in the incubator, 37°C, 5% CO_2.

   Note: Don't let the vitronectin solution dry on the wells before addition of the cell suspension.

2. Maintenance of iPSCs in culture.
   a. The StemFlex culture medium needs to be changed the next day without the ROCK inhibitor and will then be changed at least every 2–3 days.

   Note: Considerable cell death could occur and changing media helps to remove floating dead cells. Adherent cells should be visible as small colonies.

   Note: During iPSC thawing and passaging, different seeding cell densities could be tried to obtain the best density. Ideally, there should be enough distance between individual cell colonies to provide space for cell growth (see Figure 2B).

   Note: Adapt the volume of the media and timing of media change in relationship to the cell density to ensure that all iPSCs are well supported (ie: more frequent media changes are needed when there is a high number of cells in one well).

   Note: Small iPSC colonies should appear over 2–3 days with only one cell type visible. If there are multiple pre-differentiated cell types, the iPSCs need to be passaged further to obtain high quality iPSCs. See troubleshooting "problem 1".

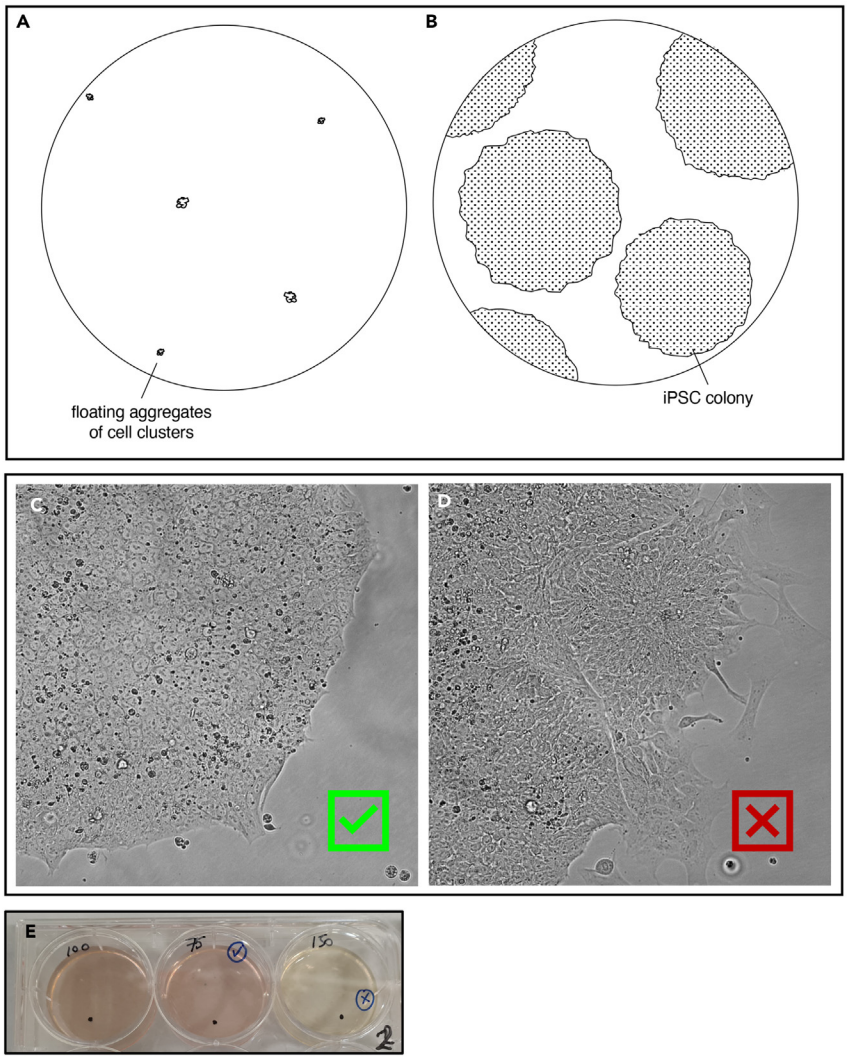

Figure 2. iPSC density

(A and B) Optimal iPSC seeding densities and confluency. Since iPSCs grow in colonies instead of single cells, it is difficult to calculate the seeding density of iPSCs using number of cells per cm^2. Here, two illustrations were drawn, looking down a brightfield microscope with a 4X objective, for references of: (A) optimal seeding density of iPSCs, showing floating aggregates of cell clusters each consisting of 5–6 cells, and (B) optimal confluency of iPSCs colonies ready for passaging. Colonies should not merge with each other and have defined edges.

(C and D) Morphology of iPSC colonies before NPC induction. Morphology of different iPSC colonies before NPC induction. Phase contrast images were taken using a FLoid imaging station. (C) A good iPSC colony with defined edges and a compact center. (C) A pre-differentiated iPSC colony with loose edges and differentiated cells, which should not be used for the NPC induction.

(E) Cell confluency affects NPCs induction. In this example, 75, 100 or 150 μL of the iPSC stock were seeded at the beginning of the induction which results at different cell density in each well. Medium is turning yellow in the well labeled "150" after 24 h of media change which indicates that the cell confluency is too high and therefore there is a risk that small molecules might be insufficient for the induction of all iPSCs inside the well. We recommend that the medium is closely monitored for color changes during NPC induction. Do not use cells from wells which have a decoloration of the medium for the NPC bank.

Note: iPSCs need to be passaged before the individual colonies merge with each other as seen in Figure 2B.

Note: It is difficult to give an optimal iPSC seeding density because they are in colonies and a count of the individual cells is an inaccurate parameter for cell seeding of colonies.

⚠ CRITICAL: It is crucial to start the NPCs induction with well-formed iPSC colonies at the optimal confluency (70%–80%). The iPSC colonies should have a compact centre and well-defined edges with no differentiated cells (see Figures 2C and 2D). Undifferentiated iPSCs are the rounded cells inside the cluster.

3. iPSC to NPC induction.
   a. Coat a 6-well plate with Matrigel (pure Matrigel solution prepared following the manufacture's instruction, see "before you begin point 1a") for 1 h at 37°C, 1 mL Matrigel each well.

   Note: Matrigel will start to gel up above 10°C, so materials and solutions need to be cold for the coating. It is recommended to incubate the plate and pipette tips in the freezer 10–15 minutes before starting the coating and to keep the Matrigel on ice.

   Note: Matrigel coating needs to be coated at 37°C at least 30 min. The coating can be done overnight (up to 16 hours) but it is not recommended to coat the plate more than 24 hours.

   b. Make up basal [RDM] and [RDMi] media according to the materials tables. Prepare 2 mL [RDMi] media for each well (6-well plate).
   c. Aspirate the media carefully in the wells with good iPSC colonies.
   d. Wash the iPSCs gently with warm DMEM-F12. Aspirate gently.
   e. Add 1 mL ReLeSR per well, and then aspirate ReLeSR after 5 s.

   Note: Make sure that ReLeSR is in contact with all the iPSC colonies. This is usually achieved by rotating the plate a few times before aspirating the ReLeSR.

   f. Start the timer for 7 min at 20–25°C. Wells are dry (without media) during this period.

   Note: It is recommended that colonies are observed under a microscope during the detachment step. Once the central cells in the colony start to break away with visible gaps, the cell monolayer is ready to be harvested. It generally takes 5–6 minutes but can fluctuate depending on the iPSC lines and the cell density.

   g. iPSCs should detach spontaneously, if not gently tap the plate.

   Note: Do not let the colonies break into single cells. At the start of NPC induction, the cells are still pluripotent and need to be in colonies/aggregates to survive (3–6 cells).

   h. Add 1 mL [RDM] basal media to each well and collect detached iPSCs colonies gently into 10 mL tubes to obtain the iPSC suspension.

   Note: Do not pipette up and down too many times (3 times at most), otherwise the colonies will disaggregate into sizes that are too small for the cells to survive. An optimal size is 3–6 cells per colony.

   i. Aspirate Matrigel and add [RDMi] media to the coated well, 2 mL per well.
   j. Add 20 μL, 50 μL or 100 μL of iPSCs suspension into each well of the coated plates, with a dilution factor of 1:50, 1:20 and 1:10, respectively. Check the cell density under the microscope.

   ⚠ CRITICAL: It is fundamental to use different dilutions of iPSC suspensions for the NPC induction to optimise the seeding density of iPSC colonies. It is critical to have the correct density of iPSC colonies seeded. The cells need to be induced for 7 days without passaging; if too many colonies are seeded, cells will rapidly exhaust the supply of small molecules present in the induction media [RDMi] by the end of the induction (days 5–7).

If insufficient colonies are seeded it can affect the cell survival. When starting with iPSC at 70%–80% confluency in the well prior to harvesting, a dilution factor of 1:20 should be optimal. However, as the extent of iPSCs disaggregation into small colonies is difficult to control, it is highly recommended to trial out different dilutions to obtain the optimal seeding density (see Figure 2E).

4. Incubate cells at 37°C, 5% CO_2, for 7 days.
5. Change media with fresh [RDMi] and small molecules every day for 7 days.

Note: Cells need to be carefully monitored, specially at days 5–7. If the media becomes yellow, it indicates that the cells might have exhausted the small molecules, with the risk of a poor NPC induction. If the cell density is not ideal, the volume of the media per well may need to be increased or changed twice a day (Figure 2E).

NPCs maintenance and creation of a NPCs cell bank

Timing: 4 days per passage

At day 7 of induction, the cells should have adopted a NPC fate, and they are ready to be passaged, expanded and frozen down to generate a cell bank for subsequent neuronal differentiation.

Note: For NPC quality control, cells can be stained with iPSC and/or NPC specific marker antibodies at different stages of induction. For a list of recommended antibodies, please refer to Wang et al. 2023.[1]

6. NPCs passage.
   a. Coat a 6-well plate with Matrigel for 1 h at 37°C, 1 mL Matrigel each well.

   Note: Matrigel needs to be coated at 37°C for at least 30 min. The coating can be done overnight (up to 16 hours) but it is not recommended to coat the plate more than 24 hours.

   b. Make up basal [RDM] and [RDMm] media according to the materials tables. Prepare 2 mL [RDMm] media for each well.
   c. Aspirate the media carefully.
   d. Wash NPCs gently with DMEM-F12. Aspirate gently.
   e. Add 0.5 mL accutase in each well.

   Note: Accutase is frozen as stock in 5 mL aliquots and should be thawed at 20–25°C. Do not thaw accutase at 37°C.

   Note: DMEM-F12 used for the washes doesn't need to be pre-warmed at 37°C.

   f. Incubate 5 min at 37°C.
   g. Add 1.5 mL -DMEM-F12 to each well.
   h. Detach the NPCs gently by tapping the plate.
   i. Pipette up and down three times and collect detached cells gently into 10-mL Falcon tubes to obtain the NPC suspension.

   Note: Pipetting up and down three times should be enough to detach the cells and generate a single cell suspension.

   Alternative: If pipetting doesn't break up the cell clusters, a cell strainer (40 μm diameter) can be used to break the cell clusters and to obtain a single cell solution.

j. For the first passage of NPCs (Induction P0 to NPC P1), dilute the cell suspension by a 1:3 dilution factor (therefore 2 mL of cell suspension obtained from each well can be expanded into 3 new wells at 2 mL per well).
k. Centrifuge cell suspension at 200 *g* for 3 min at 20–25°C.
l. Remove the supernatant and resuspend cells in [RDMm] media with small molecules.

Note: For 2 mL of cell suspension centrifuged, cells should be resuspended in 6 mL [RDMm] media and will then split into 3 different wells.

Note: For reference, NPCs can be passaged and seeded at 30,000 cells/cm^2. However, the cell survival rate of single-cell NPC suspension can fluctuate between 50%–90% depending on cell density and passage number. Hence, seeding a definitive number of NPCs may result in varied confluency of surviving NPCs. Applying the 1:3 dilution factor generally results in more consistent NPC cultures than following exact seeding numbers.

Optional: The ROCK inhibitor Y27632 can be added to the [RDMm] media to promote cell survival during NPC passaging. However, do not leave Y27632 in the media longer than 24 hours.

m. Aspirate the Matrigel and add resuspended cell suspension to the coated well, 2 mL per well.
n. Incubate the NPCs at 37°C, 5% CO_2.

7. NPCs maintenance.
   a. Change media every 2 days.
   b. NPCs monolayer are ready for the next passage at 80%–90% confluency. Repeat step 6 to expand the NPCs further.

Note: it takes 3–5 days for the NPCs to reach confluency again and to be passaged.

8. To freeze down NPCs, repeat steps 6a to 6i to detach the NPCs and collect the cell suspension.
   a. Centrifuge NPCs suspension at 200 *g* for 3 min at RT.
   b. Count NPCs and freeze 1 × 10^6 NPCs in one vial.
   c. Resuspend NPCs in CryoStor CS10 (freezing media).
   d. Transfer 500 μL of NPCs in freezing media per vial.
   e. Transfer vial in a special freezing container to allows the temperature to slowly cool down and keep at least 4 h at −80°C.
   f. Transfer NPCs frozen vial in the liquid nitrogen for longer storage.

Pause point: NPCs can be frozen and thawed when needed.

9. If thawing NPCs:
   a. Repeat steps 6a and 6b coat the plate and prepare media.
   b. Transfer the NPCs from liquid nitrogen and thaw cells quickly in a water bath at 37°C.
   c. Add 5 mL [RDM] basal media to the cells drop-wise while shaking.
   d. Centrifuge at 200 *g* for 3 min at 20–25°C.
   e. Remove the supernatant and resuspend the cell pellet in [RDMm].
   f. Aspirate the Matrigel and seed NPCs into the Matrigel-coated 6-well plates, incubate at 37°C, 5% CO_2, until the NPCs are confluent (80%–90% confluency) and ready for the next passage or to be seeded for the neuronal differentiation.

Note: It is recommended to include the ROCK inhibitor Y27632 in the [RDMm] media when thawing NPCs. Do not leave Y27632 in the media longer than 24 hours.

Note: NPCs can be expanded and frozen down from many passages. However, for an optimal neuronal differentiation, it is recommended to use NPCs passaged between P3 and P10.

Table 1. Example of different NPCs seeding calculation

Different purposes	Concentration of the NPCs suspension (count)	[Number of NPCs needed /well] x number of well	Volume of NPC suspension (before spinning)	Resuspension volume after the spinning and removing the supernatant
1 - NPCs for neuronal seeding (into three wells of a 24-well plate)	3×10^5 cells/mL	3×10^4 cells/well x 3	$(3 \times 10^4) \times 3 \div (3 \times 10^5)$ = **300 µL**	Resuspend in 1.5 mL [N2B21+] (into 3 wells of a 24-well plate, 500 µL per well)
2 - NPCs for cell maintenance (into one well of a 6 well plate)	3×10^5 cells/mL	1.2×10^5 cells/well	$(1.2 \times 10^5) \div (3 \times 10^5)$ = **400 µL**	Resuspend in 2 mL [RDMm] (into one well of a 6 well plate)
3- NPCs for freezing	3×10^5 cells/mL	9×10^5 cells/vial	$(9 \times 10^5) \div (3 \times 10^5)$ = **3 mL**	Resuspend in 1 mL in CryoSTOR into one vial cryotube

Note: For each new batch of NPCs induction, it is important to test that neurons can be generated from the NPC cell bank following after a freeze/thaw cycle.

NPC to neuron differentiation

Timing: 7–28 days

In this third step, NPCs are terminally differentiated into neurons. An established protocol has been adapted to differentiate NPCs into mature neurons[3] with modification.[4] Seeded NPCs are noted as "differentiation day 0" in the differentiation timeline. Using NPCs as the starting point generates a highly reproducible timeline for neuronal differentiation. We decided to stop the neuronal culture after 28 days, but it can be stopped at any time.

Note: Researcher should label each new procedure with the date of differentiation to maintain a comprehensive record.

10. Seeding NPCs for neuronal differentiation.
    a. Coat wells, coverslips or chamber slides with poly-L-ornithine at 20 µg/mL, 250 µL per well for 24-well plates, for at least 2 h at 37°C.

    Note: Poly L-ornithine is diluted at 1:5 in ultra-pure water at 0.01% ; ie: 1 mL of poly-L-ornithine in 4 mL of water.

    Pause point: The coating with poly-L-ornithine can be done overnight.

    b. Wash the wells or coverslips with ultrapure sterile water (or PBS) 3 times.

    Note: Make sure that wells or coverslips are dry before adding the laminin.

    c. Coat wells, coverslips or chamber slides with laminin at 20 µg/mL, 250 µL per well for 24-well plates, 37°C for at least 2 h.

    Note: Laminin is expensive, so it is recommended to calculate exactly how much is needed for the seeding according to the laminin aliquot available. One aliquot of 1 µg laminin is diluted in 5–10 mL of cold PBS, and stored at −20°C. The frozen laminin aliquot needs to be spun down few seconds and thawed on ice for 10/15 minutes. Pipette tips need to be pre-cooled in the fridge when handling laminin.

    Alternative: Matrigel can be used as a cheaper alternative to laminin with 1 mL per well (6 well plate), but neurons seeded on Matrigel tend to detach more easily than those seeded on laminin from the second week of differentiation onwards.

d. Make up basal [N2B21] media and [N2B21+] media with growth factors according to the materials tables. Prepare 250 μL [N2B21+] media per well for a 24-well plate.
e. Aspirate media on NPC monolayers carefully.
f. Wash NPC monolayers gently with -DMEM-F12. Aspirate gently.
g. Add 0.5 mL accutase to each well.
h. Incubate 5 min at 37°C.
i. Add 1.5 mL DMEM-F12 to each well.
j. Detach NPCs gently by tapping the plate.
k. Collect detached cells gently into 10-mL Falcon tubes to obtain the NPC suspension.
l. Count cells.

Note: 3×10^4 NPCs are seeded into one well of a 24-well plate, which corresponds to a cell density of 1.58×10^4 cells/cm^2. The number can be adjusted according to the purpose of the experiment (ie: seeding higher number of cells to obtain a denser neuronal network). Do not seed cells at a density lower than 1×10^4 cells per well; neurons will be too sparse and without adequate neuronal connections will affect the cell survival.

Optional: If the NPCs are to be cultured for multiple purposes (ie: neuronal differentiation, NPC maintenance and/or NPC freezing), distribute the specific volume of NPC suspension for each purpose in individual 10-mL Falcon tube. Table 1 above can be used as an example to calculate the correct volume of NPC cell suspension required for each purpose.

m. Centrifuge NPC suspension at 200 *g* for 3 min at RT.
n. Aspirate supernatant and resuspend NPCs in [N2B21+] media with small molecules and the ROCK inhibitor Y27632 (5 μL in 10 mL final).

Note: if the NPC are divided into individual Falcon tubes for different purposes, resuspend with the correct media and correct volume for each purpose (ie: N2B21 for NPCs seeding, RDM for NPCs maintenance and CryoIStor for freezing NPCs). See Table 1.

o. Aspirate laminin from the coated cultureware and add resuspended cells to the coated well, 250 μL per well for 24-well plates.

Note: To be sure that the laminin does not run dry, it is better to perform step o. well by well.

p. Incubate the NPCs at 37°C, 5% CO_2. The seeding day corresponds to neuron differentiation Day 0 and plastiware should be labeled with the date of the differentiation day/Day 0).
q. During the neuronal differentiation:
   i. Do a full media change with [N2B21+] containing fresh growth factors (without Y27632) at Day 1.
   ii. From day 1 onwards, replace half the media volume with an equivalent volume of fresh [N2B21+] containing fresh growth factors added every 2–3 days.

Note: By day 1 differentiation, NPCs should be in clusters, at 15/20% confluency. Dendrites can already be seen in presence of the ROCK inhibitor.

Note: Examine the cells daily for morphological changes. A reference timeline of cell morphology during neuronal differentiation is shown in Figure 4.

Note: The identities and development of the neurons can be validated by immunofluorescence using different NPC and/or neuronal markers at different developmental stages. For recommended antibodies working in human neurons, see Wang et al. 2023.[1]

Alternative: If NPC number is low at the beginning of the neuronal differentiation, the proliferation of NPCs can be enhanced by replacing the NBM basal medium with 50% DMEMF-12 and 50% NBM basal medium from day 1 to day 3. See troubleshooting "problem 2".

⚠ CRITICAL: Do not let the wells dry when changing for fresh [N2B21+] medium. The neuronal cell layer is highly likely to detach from the surface of the well if the laminin coating is dry during media changes. Always leave a portion of a small volume of conditioned media in the well, sufficient to cover the cells.

Note: "Conditioned media" refers to the media already used to maintain the cell culture, containing growth factors and protein secreted by the cells, present in the well.

Optional: If proliferative non-neuronal cells arise in the culture, 2 μM of 5-FdU (a thymidylate synthase inhibitor) can be added to the culture around Day 10 until fixation at Day 14 to kill proliferating cells. Alternatively, neurons can be detached and replated to physically separate the neuronal layer to non-neuronal cells from the more adherent non-neuronal cells. See troubleshooting "problem 3" and Figure 6.

Monitoring neuronal differentiation using the IncuCyte

Timing: 7–28 days

The complete timeline of the neuronal differentiation from NPCs (differentiation day 0), can be monitored with long-term live cell imaging using the IncuCyte instrument. The IncuCyte is a wide-field imaging system inside of an incubator which can image cultured cells without disturbing their environment. This protocol can be adapted for other cell imaging microscopes (i.e.,: CELLCYTE X™, Olympus Viva View FL). In this last step, a protocol is described which can record the neuronal dynamics during the different steps of the neurogenesis, as well as the dynamics of intracellular organelles, such as the Golgi apparatus (Figure 5, Methods video S1). Organelles can be followed in live cell using specific dye and/or by the transfection or transduction with fluorescent tagged-organelle markers plasmids (for reference see Wang et al.[1]).

11. Seed NPCs with [N2B21+] in culture dishes compatible with the IncuCyte system (and begin NPC differentiation to neurons as described in steps 10a to 10q). In this protocol an 8 wells iBidi μ-Slide was used for imaging.

    Note: Most of the common culture well plates or chambers are compatible with the IncuCyte. To check if a specific cultureware is supported, search in the "Vessel Type Search" page which lists all the cultureware that could be used inside the IncuCyte. If a glass-bottom cultureware is needed for high imaging resolution and/or neuronal attachment, it is recommended to use slide chambers such as an iBidi μ-Slide.

12. On day 1 of neuronal differentiation, add BODIPY TR Ceramide Golgi dye to the culture media (ready-to-use solution) at 1:1000 dilution.

    Note: Add the Golgi dye to the culture media every 2–3 days.

13. Put the iBidi μ-Slide inside the IncuCyte imaging system and clip it securely on the slide holder.

    Note: To achieve long-term imaging of the same field, it is important to secure the plates firmly on the plate holder. This is to ensure that the same position in the well is imaged at each timepoint. See troubleshooting "problem 4".

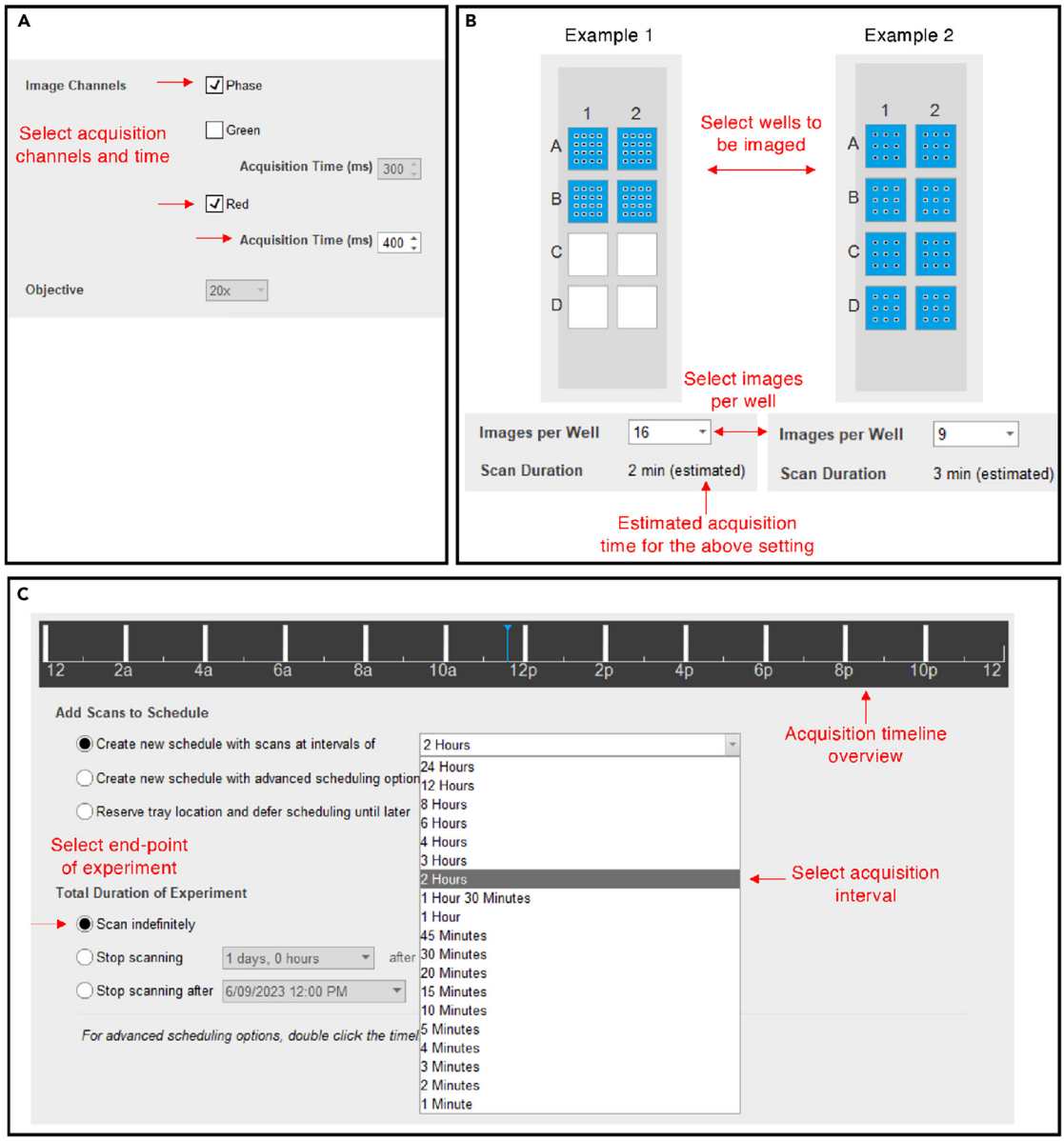

Figure 3. Set-up of the IncuCyte system at the start of the long-term imaging experiment

(A) In "Scan Settings", select objectives, acquisition channels and acquisition time. An example is shown with a 20X objective and two channels (phase contrast and Red channel) set to default acquisition time.

(B) In "Scan Pattern", select wells to be imaged and positions imaged per well. Two example patterns are shown: one with 16 positions per well and 4 wells selected (left), and one with 9 positions per well and 8 wells selected (right).

(C) In "Scan Schedule", select the scan interval/frequency and the end point of the imaging experiment. An example imaging timeline is shown to acquire images every 2 h indefinitely until the experiment is stopped.

Alternative: Other methods to identify the Golgi or other organelles can be adopted, such as transducing the neurons with fluorescently labelled organelle markers, or the use of other cell painting reagents such as the lysotracker or mitotracker.

14. Set-up the parameters for imaging following the prompt from the system (Figure 3):

Note: The IncuCyte scans cultured cells on a selected schedule (e.g. every hour) which can continue indefinitely as long as the cells to be imaged are healthy (up to 28 days in this

protocol). Each scan will take a defined time, and the maximum duration for a single scan need to be half of the acquisition time interval (ie: to scan every 30 minutes, the duration for a single scan cannot exceed 15 minutes). It is important to set up the scan time and frequency so that it is within the limit but maximised to generate sufficient data for the purpose of the experiments. The duration of a single scan depends of: (1) number of channel used, (2) duration of the acquisition for each channel, (3) number of chambers, (4) number of wells imaged, and (5) number of positions or fields imaged per well, which is explained in the following steps.

a. In "Scan Settings", select objective, acquisition channels and acquisition time (Figure 3). Here, a 20X objective is used with two channels (phase contrast for neuronal morphology and RFP for Golgi dye) with default acquisition time (400 ms).

Note: The default acquisition time (300 ms for the green channel, 400 ms for the red channel) works well for most experiments. The acquisition time can be increased for dim signals, but this risks phototoxicity and prolonged scanning time for each scan. Phenol red in culture medium does not generate any noticeable effect on the red channel. If phenol red generates autofluorescence on the green channel, phenol-free media should be used.

b. In "Scan Pattern", select wells to be imaged and positions imaged per well (for pattern examples see Figure 3). In this protocol, 16 positions/fields imaged per well were imaged for the 8 wells of the iBidi μ-slide.

Note: The more wells imaged and/or more positions imaged per well, the longer the duration for each scan will be. A "scan duration" is estimated at this set-up step for the pattern chosen (Figure 3). The scan duration should not exceed half of the acquisition interval/frequency.

c. In "Scan Schedule", select the scan interval/frequency (Figure 3). Set up the end point of the imaging experiment as well (Figure 3). In this protocol, cells are imaged every 30 min for up to 28 days.

Note: The default scanning schedule starts on the hour (i.e: scanning will start exactly at 10 am, 11 am, 12 pm, if chosen to image every hour). This can be changed by dragging the timeline as seen in Figure 3, but it is recommended to follow the default schedule so that it is clear at what time the IncuCyte is imaging. This is convenient for changing media for the cells (see step 15).

Note: It is recommended to select "Scan indefinitely" (see Figure 3). The experiment can be stopped at any time by deleting the schedule. The IncuCyte will continue scanning the slide until the experiment is deleted.

15. Change the media every 2/3 days as you would do for neurons cultured in the incubator.

Note: Only change media when the IncuCyte is not imaging/operating. The available time to change media can be calculated by the scanning duration and frequency. For example, if (1) the IncuCyte is imaging every 30 minutes, (2) each scan takes 7 minutes, and (3) the scan starts exactly on the hour; then the IncuCyte will be imaging from 10:00 to 10:07 am, and a media change can be performed from 10:08 to 10:29 am.

16. Check the status of the cells and the IncuCyte regularly during the long-term imaging to make sure neurons are healthy and the imaging settings are good.

Note: In the middle of an imaging experiment, scans already done are accessible anytime in the "View" function of the IncuCyte software.

Note: After two weeks of differentiation, neurons can be in a 3D cluster instead of a monolayer, in which case they can be detached and re-seeded in a new coated slide and the analysis continued. See troubleshooting "problem 5".

Note: Having a duplicate neuron plate in the cell culture incubator (not the IncuCyte incubator) can be a good quality control and a backup in case of any technical fault associated with collection of images during the IncuCyte experiment.

17. Export the image series from the IncuCyte when the imaging is finished.
18. Analyze the image series using the image analysis software according to the requirements of the experiment.

Note: When exporting data, it is recommended to choose the option to export images "as stored". This will export the raw images for each timepoint with full image quality. In this protocol, raw tif images were reconstructed using the software FIJI/ImageJ to a time-series using the "images to stack" function.

Alternative: Sartorius offers various automated analysis modules, which include neuronal tracking and growth analysis. These modules are available for purchase if required (see Sartorius.com).

EXPECTED OUTCOMES

The protocol presented here is used to monitor the dynamics of the Golgi apparatus in human iPSC-derived neurons during differentiation. Following these three steps: 1) iPSC to NPC induction, 2) NPC to neuron differentiation and 3) IncuCyte live-cell imaging, a movie of a functional neuronal population should be obtained, with a reproducible differentiation timeline from NPCs consisting of 3 stages (Figure 4, Methods video S1): (1) day 0 to 3, NPC to neuron polarization with establishment of the neuronal morphology; (2) day 3 to 10, active neuronal migration in a monolayer with neurite outgrowth; (3) day 10 onwards, formation of neuronal network and clusters with axon and dendrite polarization. The long-term live cell imaging technique allows powerful visualization of the distinct dynamics and behavior of neurons during different stages of neuronal differentiation, which is difficult to observe using fixed cells or short-term imaging techniques.

Qualitative and/or quantitative analyses can be obtained from different stages of neurogenesis, as required for the specific aims of the experiments. For instance, Sartorius (the developer of the IncuCyte instrument) proposed a range of quantitative tools on neuronal analysis, including but not limited to : (1) quantification of neuronal networking by measuring the density of neurite growth, (2) quantitative measurement of neuronal activities by illuminating Calcium influx and efflux, and (3) fluorescence quantification of specific tagged proteins of interest (see Sartorius.com).

In this protocol, the long-term live cell imaging technique enables visualization of the spatial-temporal dynamics of the Golgi apparatus during differentiation. In healthy iPSC-derived neurons, the Golgi has distinct behaviors during different stages of neuronal development (Figures 4 and 5); the entire Golgi is transiently translocated to the developing neurite during migration, and stable, discrete dendritic Golgi outpost structures are formed at a later developmental stage. The characterization of these dynamic Golgi events has been reported in detail in Wang et al. (2023).[1]

The protocol described herein can be applied to patient-derived neurons to characterize and compare the neuronal dynamics in developing and functional neurons as well as used for drug screening. In addition, the protocol can be expanded to characterize other organelles, such as Golgi, ER, endosomes and mitochondria, in combination with techniques to illuminate specific compartments in live cells. Furthermore, molecular mechanisms could be studied in human neurons by

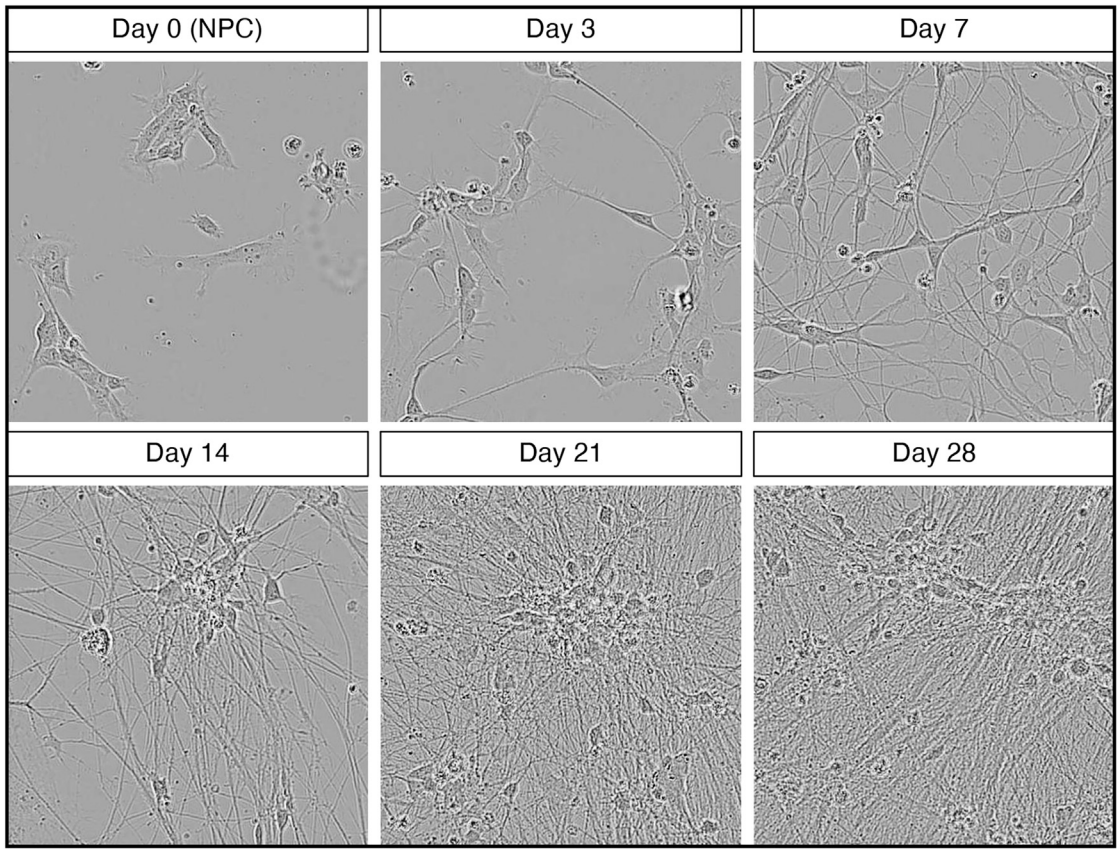

Figure 4. Cell morphology during neuronal differentiation

Cell morphology during the differentiation of NPCs from day 0 into neurons (day 14). Phase contrast images were taken using the IncuCyte. Days 0–3, NPCs are polarized with protruding neurites; days 3–10, developing neurons actively migrate to establish connections; day 10 onwards, neurons organize themselves into 3D clusters.

manipulating gene expression and/or protein levels followed by live-cell monitoring. The long-term live cell imaging technique can provide insight into the organization of the secretory network in human iPSC-derived neurons in heath and disease.

LIMITATIONS

One limitation in the neuronal differentiation protocol described is that neurons naturally tend to migrate and cluster together into 3D spheres after two weeks of differentiation. The formation of a complex and extensive neuronal network makes single neuron analysis difficult at late stage of differentiation. This limitation can be overcome by detaching the cells and replating the neurons on a new plate and/or by guiding the single cell neuronal network formation using micropatterning.

Another limitation is the amount of data rapidly generated by the IncuCyte and it can be time consuming and challenging to analyze every field generated.

But the major limitation of the IncuCyte is the inability to image z-stacks and to analyze 3D neuronal structures (from day 21), and the low resolution. To overcome the last two limitations, confirmation of interesting IncuCyte data can be performed for selected time points with high quality live cell confocal microscopy (i.e., spinning disk) for 3D imaging at a higher resolution.

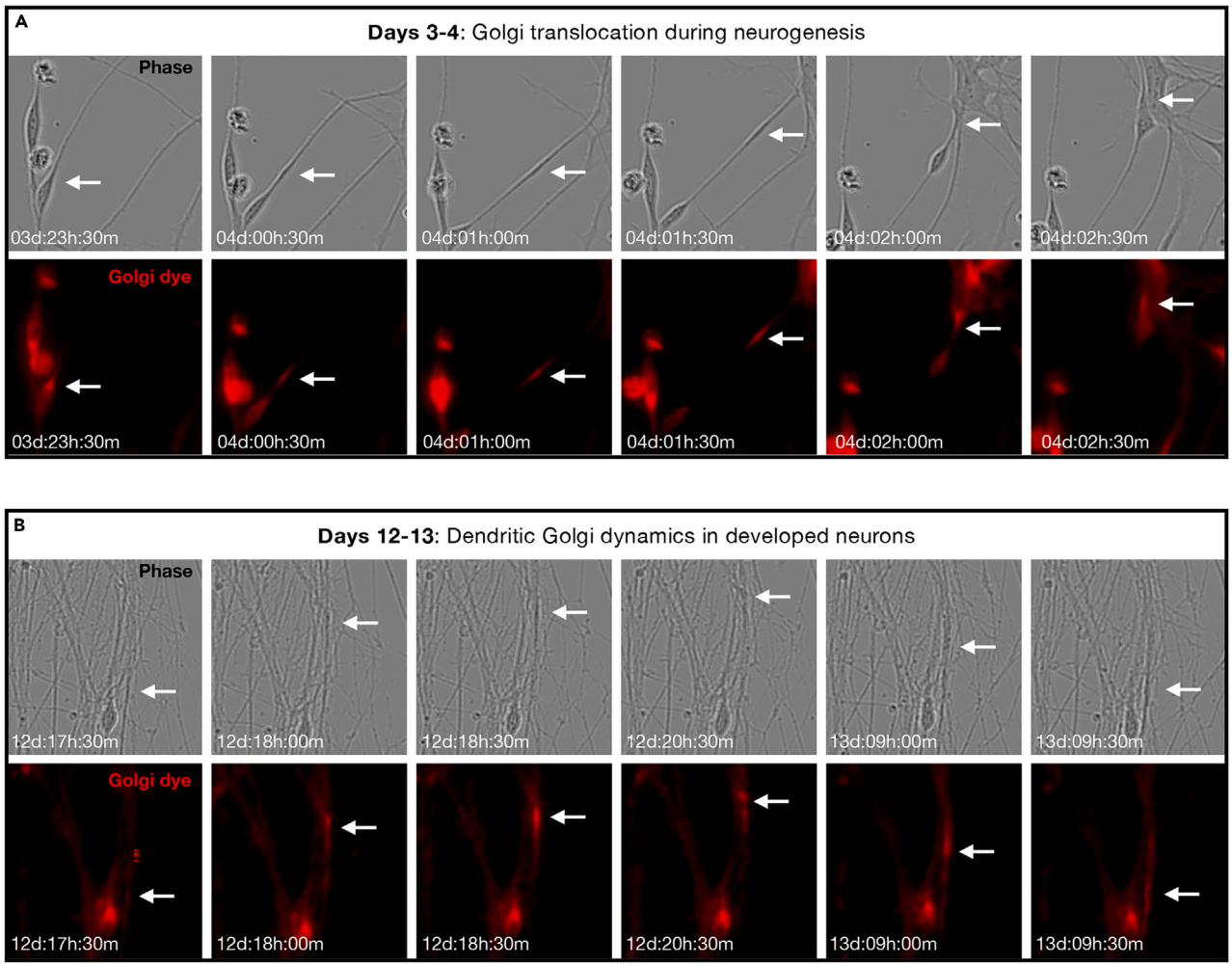

Figure 5. Dynamics of dendritic Golgi monitored during the neuronal development

(A and B) Live human neurons were incubated with the red ceramide Golgi dye, BODIPY TR Ceramide, and imaged every 30 min using the IncuCyte instrument from differentiation day 1 to 21. Phase contrast and fluorescent images were taken at indicated time. Arrows indicate Golgi structures in neurites of live neurons. (A) Golgi translocation during neuron migration (differentiation days 3–4). (B) Dendritic Golgi dynamics in developed neurons (differentiation days 12–13).

TROUBLESHOOTING

Problem 1

There are pre-differentiated cells in the iPSC culture before iPSC to NPC induction (Step 2a).

Potential solution

It is crucial to start iPSC to NPC induction with iPSC colonies of good quality. If loose, pre-differentiated cells are observed at the edge of the iPSC colonies, iPSCs can be passaged with ReLeSR to obtain purer colonies. With optimized detaching time (5–7 min), ReLeSR will only detach undifferentiated iPSCs from the center of the colonies, leaving the unwanted, pre-differentiated cells still attached during passaging. This way, iPSCs can be "purified" for a few passages to obtain high quality iPSC population. Alternatively, spontaneous differentiating cells can be scraped off. Passaging iPSCs 2–3 times with ReLeSR should be sufficient to improve iPSC quality.

Problem 2

The number of NPCs is insufficient at the beginning of the neuronal differentiation (step 10).

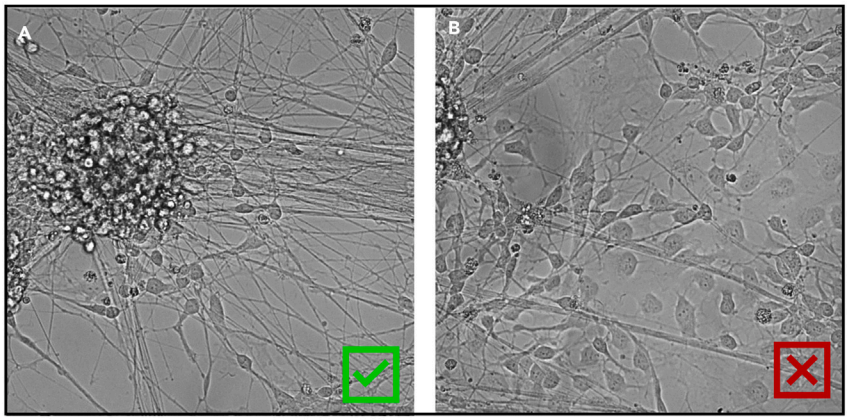

Figure 6. Successful and unsuccessful neuronal differentiation

(A and B) Neuronal morphology at differentiation day 14. Phase contrast images were taken using the Floid imaging station. (A) A good differentiated cluster of neurons with a high percentage of cells adopting the neuronal fate. (B) An unsuccessful neuronal differentiation with non-neuronal cell predominating.

Potential solution

- If NPC numbers appears to be low at the beginning of the neuronal differentiation, the proliferation of NPCs can be enhanced by changing the basal medium of [N2B21] from 100% NBM to 50% DMEM/F-12 and 50% NBM from day 1 to day 3. That is, for 50 mL of [N2B21], instead of using 48 mL NBM as the basal medium, use 24 mL NBM and 24 mL DMEM/F12. Note that change affects only this basal medium change, neuronal growth factors (BDNF, GDNF, cdb-cAMP, AA and C-E) are still added and the cells are signaled to start differentiation from the seeding day (day 0), hence the seeding day should still be labeled as "differentiation day 0". The media is then gradually switched to [N2B21] with 100% NBM from day 4 by half media changes.
- For extreme cases where the NPCs do not look healthy enough at the start of the differentiation, the culture medium can be switched to [RDMm] for 1–3 days to promote NPC expansion. Note that this way, neuronal growth factors (BDNF, GDNF, cdb-cAMP, AA and C-E) are not present in the media for these days, instead, small molecules to maintain NPC identity (CHIR, SB and LIF) are present. After 1–3 days, the media can be switched to [N2B21] to initiate neuronal differentiation. The day when [RDMm] is switched to [N2B21] should be denoted as "differentiation day 0" instead of the seeding day.

Problem 3

There are non-neuronal cells in the differentiated neuronal population, interfering with the imaging analysis (Step 10).

Potential solution

- A critical point is to make sure that there is no exhaustion of the small molecules throughout the NPC induction period (specifically for the last days) to be able to subsequently obtain a highly enriched neuronal population (>90% neurons in the cell population) (see Figure 4). The neuronal population should be assessed using morphological features as well as immunostaining with NPC and neuronal specific markers (see Wang et al. 2023[1] for example). If less than 70% of the cell population are neurons following the neuronal differentiation (example in Figure 6), it is highly likely that the 7-day NPC induction process (step 1) was not optimum. Therefore, if it is the case it is recommended to re-start a new NPCs induction with a lower seeding density of iPSCs, and with more frequent changes of the [RDMi] media to make sure that the quantity of small molecules

is sufficient for the chemical-induced differentiation for the iPSC/NPC population. Of note, having insufficient iPSC cells at the beginning of the neuronal differentiation can also negatively affect the NPC induction and therefore the neuronal differentiation.

- If there are 80%–90% of neurons in the population following differentiation, it is possible to increase the percentage of neurons in the culture by including 2 μM of thymidylate synthase inhibitor (5-FdU) in the culture medium. A low level of 5-FdU (2 μM) kills proliferative cells but because well-differentiated neurons are post-mitotic, they are resistant. Note that there will be cell death of the non-neuronal cells, and cell debris can be removed from the culture by regular and gentle half media changes.
- In addition to the inclusion of 5-FdU, neurons can be physically separated from the non-neuronal population by selective detachment and then replating. In general, non-neuronal cells are larger in size and tend to attach more strongly to the surface of the culture dish, and neurons are smaller and easier to detach. They can be detached using accutase (see steps 10e–10i). A shorter incubation time with accutase (1 min) will detach the neuronal layer from the non-neuronal layer sitting underneath (the neurons are still in a network instead of dissociated single cells), while non-neuronal cells will remain attached to the cultureware. In this way, the neuronal population can be selectively replated. Replating should be done gently to avoid dissociating neurons into single cells; see problem 2. Replating neurons in combination with the addition of 2 μM 5-FdU in the culture media can achieve a purify of >98% neurons in cell culture, if desired.

Problem 4

The imaging field of IncuCyte shifts or moves slightly during each image acquisition (Step 13).

Potential solution

Always make sure that the plate is firmly clipped into the IncuCyte holder. If the imaging field shifts/move during the acquisition, movies can be re-aligned during the image analysis and online algorithms are available. We use the FIJI/ImageJ Plugins called "registration – linear stack alignment with SIFT" to re-align the frames when there is a slight shifting of positions during the image acquisition.

Problem 5

Neurons are organized in 3D clusters from day 10 of differentiation, and single cells are hard to observe using the IncuCyte (Step 16).

Potential solution

Neurons can be detached using accutase and replated (see problem 3, third bullet point). 3D neuronal clusters, after being detached and in suspension, can be gently pipetted to disaggregate the clusters into smaller sizes and replated onto newly-coated surfaces. However, it is important to note that the clusters should not be disaggregated into single cells. Neurons, especially at a later stage of differentiation (>day 14), rely on networking for correct physiology and survival. Breaking connections between neurons can lead to cell death. Generally, a minimal of 5–6 neurons per cluster is required for the replated neurons to survive. Conditioned media from the culture can be kept and added to the replated neurons to increase survival. It is estimated that around 80% of neurons will survive after detaching and replating.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof Paul A. Gleeson (pgleeson@unimelb.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The data reported in this protocol will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102699.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.W., P.A.G., L.F.; writing - original draft, J.W., L.F.; writing - reviewing and editing, J.W., P.A.G., L.F.; visualization, J.W.; supervision, P.A.G., L.F.; funding acquisition, P.A.G., L.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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